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(54) Title: LABELLED GLUTAMINE AND LYSINE ANALOGUES

(57) Abstract

Synthetic analogues of lysine and glutamine are provided which function as substrates for the fibrin-stabilising enzyme Factor XIIIa even when labelled with a detectable moiety. The use of suitable protecting groups provides compounds which possess reduced susceptibility to *in vivo* metabolism especially by peptidases, and are hence useful agents for the diagnosis of thrombosis, embolism, atherosclerosis, inflammation or cancer.

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LABELLED GLUTAMINE AND LYSINE ANALOGUES

5 The present invention relates to a class of compounds useful in the diagnosis of sites of venous and arterial thrombosis, embolism or infection, pharmaceutical formulations containing them, their use in the diagnosis of disease and methods for their preparation.

Prior approaches to thrombus imaging radiopharmaceuticals include radiolabelled fibrinogen or plasminogen; radiolabelled fragment E, 10 of human fibrin; radiolabelled plasminogen activators such as tissue plasminogen activator (t-PA) and labelled anti-fibrin antibodies. Methods based on the detection of sites of platelet accumulation such as the administration of radiolabelled platelets (e.g. using ^{111}In oxine) or radiolabelled anti-platelet antibodies have also been described. More 15 recent efforts have focused on radiolabelled peptides or polypeptides such as the cell adhesion motif RGD (where R, G and D are the standard abbreviations for the amino acids arginine, glycine and aspartic acid respectively); platelet factor 4 or fragments thereof or anticoagulant peptides such as disintegrins.

20 Factor XIII is a plasma glycoprotein which is present in blood and certain tissues in a catalytically inactive (or zymogen) form. Factor XIII is transformed into its active form Factor XIIIa by thrombin in the presence of calcium ions. Factor XIIIa is also known as plasma transglutaminase, fibrinoligase or fibrin-stabilising factor. The final step in the formation of a 25 blood clot is the covalent crosslinking of the fibrin which is formed by the proteolytic cleavage of fibrinogen by thrombin. Fibrin molecules align and the enzyme Factor XIIIa catalyses covalent crosslinking of the NH_2 and CONH_2 groups of lysyl and glutamyl residues respectively giving structural rigidity to the blood clot. The crosslinking stabilises the fibrin clot 30 structure and confers resistance to fibrinolysis. The crosslink formation is

an important facet of normal blood coagulation and wound healing as well as pathological conditions such as thrombosis. As atherothrombotic brain infarctions are a common sub-type of stroke, Factor XIIIa substrates may allow diagnosis of stroke. It may also be implicated in atherosclerosis, 5 inflammatory processes, tumour growth and metastasis. WO 91/16931 discloses that radiolabelled analogues of Factor XIII (in which the active site has been inactivated by amino acid substitution) are useful as thrombus imaging radiopharmaceuticals.

Factor XIIIa is also known to catalyse the incorporation of low 10 molecular weight amines into the γ -glutamine sites of proteins. Similarly Factor XIIIa also catalyses the incorporation of low molecular weight glutamine analogues into lysyl residues. Thus such low molecular weight amines (or glutamine analogues) function as competitive inhibitors of the Factor XIIIa-induced lysyl/glutaminyl crosslinking of proteins. 15 A range of synthetic amines have been described which are competitive inhibitors of the uptake of labelled putrescine (1,4-butanediamine) into N,N'-dimethylcasein catalysed by pig liver transglutaminase [L.Lorand et al., Biochem., 18, 1756(1979)].

WO 89/00051 (Cytrix Biopool Ltd.) claims a method for 20 targeting fibrin deposits using a labelled compound which is covalently bound to fibrin by Factor XIIIa. The fibrin binding compound is stated to be "any peptide that is a substrate for the blood enzyme commonly known as Factor XIIIa". Preferred peptides are said to include the tetrapeptide sequence -Asn-Gln-Glu-Gln- (or NQEQ in standard amino acid 25 abbreviation notation). Also disclosed is the 12-mer peptide sequence from the NH₂ terminus of the alpha-2 antiplasmin enzyme: NH₂-Asn-Gln-Glu-Gln-Val-Ser-Pro-Leu-Thr-Leu-Thr-Leu-Leu-Lys-OH together with a synthetic analogue: NH₂-Asn-Gln-Glu-Gln-Val-Ser-Pro-Tyr-Thr-Leu-Thr-Leu-Leu-Lys-OH, 30 (denoted NQEQQSPLTLTLLK and NQEQQVSPYTLTLLK respectively). The

latter was radiolabelled with ^{125}I and shown to be taken up in thrombin clots *in vitro*.

It has now been discovered that synthetic analogues of lysine and glutamine labelled with a suitable detectable moiety can also function 5 as substrates for the enzyme Factor XIIIa. The use of suitable protecting groups provides compounds which are less susceptible to *in vivo* metabolism especially by peptidases, and are hence more useful targeting agents.

The present invention provides the following compounds:

10 $\text{Y}-(\text{CR}_2)_n-\text{X}-\text{NHJ}$

where:

X is $\text{C}=\text{O}$ or CR_2 ;

n is an integer of value 1 to 6;

15 Y is $\text{L}(\text{A})_m-$ or $\text{R}^1\text{R}^2\text{CR}-$ where L is a metal complexing agent, A is $-\text{CR}_2-$, $-\text{CR}=\text{CR}-$, $-\text{C}\equiv\text{C}-$, $-\text{NRCO}-$, $-\text{CONR}-$, $-\text{SO}_2\text{NR}-$, $-\text{NRSO}_2-$, $-\text{CR}_2\text{OCR}_2-$, $-\text{CR}_2\text{SCR}_2-$, $-\text{CR}_2\text{NRCR}_2-$, a C_{4-8} cycloheteroalkylene group, a C_{4-8} cycloalkylene group, a C_{5-12} arylene group, a C_{3-12} heteroarylene group or a polyalkyleneglycol, polylactic acid or polyglycolic acid moiety;

20 m is an integer of value 0 to 10;

where one of R^1 and R^2 is $-\text{NH}(\text{B})_p\text{Z}^1$ and the other is -

25 $\text{CO}(\text{B})_q\text{Z}^2$ where

p and q are integers of value 0 to 45, and

each B is independently chosen from Q or an amino acid,

where Q is a cyclic peptide;

25 Z^1 and Z^2 are protecting groups;

J and each R group are independently chosen from H , C_{1-4} alkyl, C_{1-4} alkenyl, C_{1-4} alkynyl, C_{1-4} alkoxyalkyl or C_{1-4} hydroxyalkyl; with the provisos that:

30 (i) the total number of amino acid residues in the R^1 and R^2 groups does not exceed 45;

- (ii) when X is CR₂, then Y is -CRR' R² and Z² is a metal complexing agent;
- (iii) when Y is -CRR' R² then at least one of R¹ and R² bears at least one detectable moiety.

5 The invention also includes kits for the preparation of the above compounds labelled with a detectable moiety, and the use of these and related compounds in the diagnosis or therapy of thrombosis, embolism, atherosclerosis, inflammation or cancer.

10 By the term "cyclic peptide" is meant a sequence of 5 to 15 amino acids in which the two terminal amino acids are bonded together by a covalent bond which may be a peptide or disulphide bond or a synthetic non-peptide bond such as a thioether, phosphodiester, disiloxane or urethane bond.

15 By the term "amino acid" is meant an L- or D-amino acid, amino acid analogue or amino acid mimetic which may be naturally occurring or of purely synthetic origin, and may be optically pure, i.e. a single enantiomer and hence chiral, or a mixture of enantiomers.

20 Preferably the amino acids of the present invention are optically pure. By the term "amino acid mimetic" is meant synthetic analogues of naturally occurring amino acids which are isosteres, i.e. have been designed to mimic the steric and electronic structure of the natural compound. Such isosteres are well known to those skilled in the art and include but are not limited to depsipeptides, retro-inverso peptides, thioamides, cycloalkanes or 1,5-disubstituted tetrazoles [see M. Goodman, *Biopolymers*, 24, 137, 25 (1985)].

25 By the term "protecting group" is meant a biocompatible group which inhibits or suppresses *in vivo* metabolism of the peptide or amino acid at the amino or carboxyl terminus. Such groups are well known to those skilled in the art and are suitably chosen from, for the amine terminus (Z¹): acetyl, Boc (where Boc is *tert*-butyloxycarbonyl), Fmoc

(where Fmoc is fluorenylmethoxycarbonyl), benzyloxycarbonyl, trifluoroacetyl, allyloxycarbonyl, Dde [i.e. 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl], Npys (i.e. 3-nitro-2-pyridine sulfenyl), or a metal complexing group; and for the carboxyl terminus (Z²): a carboxamide, *tert*-butyl ester, benzyl ester, cyclohexyl ester, amino alcohol or a metal complexing group. Preferably the protecting group is a metal complexing group, most preferably it is a metal complexing group bound to a metal i.e. a metal complex. The carboxyl terminus of peptides is particularly susceptible to *in vivo* cleavage by carboxypeptidase enzymes.

Consequently, the metal complexing group or metal complex is preferably attached at the carboxyl terminus. When either R¹ is -NH(B)_{p,1}QZ¹ or R² is -CO(B)_{q,1}QZ² then the protecting group may be the covalent bond which closes the cyclic peptide (Q) ring.

A "detectable moiety" is a moiety which emits a signal or is suitable for diagnostic imaging of the human body and may be a radioisotope for radiopharmaceutical imaging or therapy, a paramagnetic metal or species for MRI contrast imaging, a radiopaque group or metal for X-ray contrast imaging, a gas microbubble ultrasound contrast agent or a suitable dye for detection by external light imaging. Preferably, the imaging moiety is a metal ion, most preferably it is a radiometal.

When Y is -CRR¹R², then preferably either one or both of R¹ and R² comprises one or more peptide fragments of α_2 -antiplasmin, fibronectin or beta-casein, fibrinogen or thrombospondin. Such peptide fragments comprise at least 3 and preferably 4 – 20 amino acid residues.

When Y is -CRR¹R² and -(CR₂)-X-NHJ is -(CH₂)₄NH₂ (i.e. the amino acid side chain of lysine), then preferably either one or both of R¹ and R² comprises one or more such peptide fragments of α_2 -antiplasmin, fibronectin or beta-casein. The amino acid sequences of α_2 -antiplasmin, fibronectin, beta-casein, fibrinogen and thrombospondin can be found in the following references: α_2 -antiplasmin precursor [M.Tone *et al.*,

J.Biochem, 102, 1033, (1987)]; beta-casein [L.Hansson *et al*, Gene, 139, 193, (1994)]; fibronectin [A.Gutman *et al*, FEBS Lett., 207, 145, (1996)]; thrombospondin-1 precursor [V.Dixit *et al*, Proc. Natl. Acad. Sci., USA, 83, 5449, (1986)]; R.F.Doolittle, Ann. Rev. Biochem., 53, 195, (1984).

5 Preferably the amino acid sequence is taken from the N-terminus of:

(i) α_2 -antiplasmin,

i.e. $\text{NH}_2\text{-Asn-Gln-Glu-Gln-Val-Ser-Pro-Leu-Thr-Leu-Thr-Leu-Leu-Lys-OH}$
or variants of this in which one or more amino acids have been exchanged,
10 added or removed such as:

$\text{NH}_2\text{-Asn-Gln-Glu-Gln-Val-Ser-Pro-Leu-Thr-Leu-Thr-Leu-Leu-Lys-Gly-OH}$,
 $\text{NH}_2\text{-Asn-Gln-Glu-Ala-Val-Ser-Pro-Leu-Thr-Leu-Thr-Leu-Leu-Lys-Gly-OH}$,
 $\text{NH}_2\text{-Asn-Gln-Glu-Gln-Val-Gly-OH}$; or

(ii) casein

15 i.e. $\text{Ac-Leu-Gly-Pro-Gly-Gln-Ser-Lys-Val-Ile-Gly}$.

When the compound of the present invention is a peptide, i.e. Y is $\text{R}^1\text{R}^2\text{CR}$ - the number of amino acid residues is preferably 2 to 30, most preferably 3 to 20, especially 3 to 15.

Preferred compounds have J equal H, i.e. terminate in an
20 NH_2 group. X is preferably C=O , i.e. compounds of formula $\text{Y-(CR}_2\text{)}_n\text{-CONH}_2$ are preferred. Most preferred compounds are of formula $\text{Y-(CR}_2\text{)}_x\text{-(CH}_2\text{)}_2\text{CONH}_2$ or $\text{Y-(CR}_2\text{)}_y\text{-(CH}_2\text{)}_4\text{NH}_2$ where x is an integer of value 0 to 4, and y is an integer of value 0 to 3. Compounds having the same side chain as glutamine, i.e. glutamine analogues of formula
25 $\text{Y-(CR}_2\text{)}_x\text{-(CH}_2\text{)}_2\text{CONH}_2$ are especially preferred.

Suitable non-metallic radioisotopes for use in the present invention include but are not limited to: radioiodine such as ^{123}I , ^{125}I , ^{131}I , preferably ^{123}I ; positron emitters such as ^{18}F , ^{11}C or ^{75}Br and isotopes for therapy e.g. ^{211}At .

30 The compounds of this invention which comprise metal

complexing agents preferably have only a single type of targeting molecule attached, i.e. the $-(CR_2)_n-X-NHJ$ substituent. Other substituents on the complexing agent may be present, but the $-(CR_2)_n-X-NHJ$ substituent is the one which is expected to be primarily responsible for the biolocalisation properties. Metal complexes of the present invention may contain one or more metal ions which may be the same or different. Thus in some circumstances polynuclear complexes may have advantageous properties such as certain metal clusters which have superparamagnetic properties and are hence particularly useful as MRI contrast agents. Preferred metal complexes of the present invention involve only a single metal ion. When the metal of the metal complex is a radiometal, it can be either a positron emitter (such as ^{68}Ga or ^{64}Cu) or a γ -emitter such as $^{99\text{m}}\text{Tc}$, ^{111}In , $^{113\text{m}}\text{In}$ or ^{67}Ga . Suitable metal ions for use in MRI are paramagnetic metal ions such as gadolinium(III) or manganese(II). Most preferred radiometals for diagnostic imaging are γ -emitters, especially $^{99\text{m}}\text{Tc}$. Metal complexes of certain radionuclides may be useful as radiopharmaceuticals for the radiotherapy of various diseases such as cancer or the treatment of thrombosis or restenosis. Useful radioisotopes for such radiotherapeutic applications include: ^{90}Y , ^{89}Sr , ^{67}Cu , ^{186}Re , ^{188}Re , ^{169}Er , ^{153}Sm and ^{198}Au .

Whichever metal complex is chosen, it is strongly preferred that it is bound to the Factor XIIIa substrate in such a way that it does not undergo facile metabolism in blood with the result that the metal complex is cleaved from the Factor XIIIa substrate before the labelled Factor XIIIa substrate reaches the desired *in vivo* site to be imaged. The Factor XIIIa substrate is therefore preferably covalently bound to the metal complexes of the present invention.

These metal ions are complexed using a metal complexing agent, or more preferably a chelating agent. The chelating agents comprise 2-10 metal donor atoms covalently linked together by a non-coordinating backbone. Preferred chelating agents have 4-8 metal donor

atoms and have the metal donor atoms in either an open chain or macrocyclic arrangement or combinations thereof. Most preferred chelating agents have 4-6 metal donor atoms and form 5- or 6-membered chelate rings when coordinated to the metal centre. Such polydentate and/or macrocyclic chelating agents form stable metal complexes which can survive challenge by endogenous competing ligands for the metal *in vivo* such as transferrin or plasma proteins. Alternatively, it is possible to use monodentate complexing agents that form stable complexes with the desired metal ion even though they do not form chelate rings upon metal coordination. Examples of known complexing agents of this kind, which are particularly suitable for use with ^{99m}Tc , are hydrazines, phosphines, arsines, or isonitriles.

Examples of suitable chelating agents are bidentate such as diamines or diphosphines, tridentate such as monoaminedithiols, or 15 tetridentate such as diaminedioximes (US 4615876) or such ligands incorporating amide donors (WO 94/08949); the tetridentate ligands of WO 94/22816; N_2S_2 diaminedithiols, diamidedithiols or amideaminedithiols; N_3S thioltriamides; N_4 ligands such as tetraamines, macrocyclic amine or amide ligands such as cyclam, oxocyclam (which forms a neutral technetium complex) or dioxocyclam; or dithiosemicarbazones. The above described ligands are particularly suitable for technetium, but are useful for other metals also. Other suitable ligands are described in Sandoz WO 91/01144, which includes ligands which are particularly suitable for indium, yttrium and gadolinium, especially 20 macrocyclic aminocarboxylate and aminophosphonic acid ligands. Ligands which form non-ionic (i.e. neutral) metal complexes of gadolinium are known and are described in US 4885363. The ligand may also comprise a short sequence of amino acids such as the Cys/amino acid/Cys tripeptide of WO 92/13572 or the peptide ligands described in 25

30 EP 0719790 A2.

Preferred chelating agents have the formula



5 where each a is 2 or 3
each b is 1 or 2
one R is aminoalkylene through which the chelating agent is
joined to the rest of the molecule
each other R is independently H, C₁ – C₁₀ hydrocarbyl,
10 alkoxy, alkoxyalkyl, amine, amide, hydroxyl, hydroxyalkyl or carboxylate or
two R groups taken together with the atoms to which they are attached
form a carboxylic, heterocyclic saturated or unsaturated ring.

Non-peptide-based metal chelating agents provide improved
control over attachment and release of metal ions, and are preferred.

15 The invention also includes a peptide fragment of α_2 -
antiplasmin, fibronectin, beta-casein, tetanus, amyloid, trappin or
polyglutamine, said peptide fragment containing 3 – 45 amino acid
residues and carrying a terminal metal complexing agent.

It is well known to prepare chelating agents which have
20 attached thereto a functional group ("bifunctional chelates"). Functional
groups which have been attached to chelating agents include: amine,
carboxylic acid, cyanate, thiocyanate, maleimide and active ester such as
N-hydroxysuccinimide. Examples of chelate-amine conjugates for
diaminedioxime ligands are given in WO 95/19187. When the desired
25 Factor XIIIa substrate functionality is an amine, the ligands of the present
invention can be prepared by reaction of a bifunctional compound which
contains both an amine group (preferably protected by use of suitable
protecting groups known to those skilled in the art), and a reactive group
such as a sulphonyl chloride, acid chloride, active ester or an alkyl/benzyl
30 halide. The reactive group can then be coupled to either the pendant

amine group of a bifunctional chelate, or used to derivatise one or more of the amine donor atoms of a N-containing ligand. Alternatively, a mono-protected diamine could be reacted with a bifunctional chelate with a pendant active ester or carboxyl group to give the protected amine group linked to the ligand system *via* an amide bond. In both synthetic routes outlined above, the resulting ligand-protected amine conjugate is then deprotected under suitable conditions to give the desired amine-functionalised ligand. When the desired Factor XIIIa substrate functionality is a carboxamide group, the desired ligands can be prepared e.g. by reaction of a omega-haloalkyl carboxamide of suitable chain length with a bifunctional chelate with a pendant amine group, giving the desired carboxamide-linked ligand.

The metal complexes of the present invention may be prepared by reacting a solution of the metal in the appropriate oxidation state with the ligand at the appropriate pH. The solution may preferably contain a ligand which complexes weakly to the metal (such as chloride, gluconate or citrate) i.e. the metal complex is prepared by ligand exchange or transchelation. Such conditions are useful to suppress undesirable side reactions such as hydrolysis of the metal ion. When the metal ion is ^{99m}Tc , the usual starting material is sodium pertechnetate from a ^{99}Mo generator. Technetium is present in ^{99m}Tc -pertechnetate in the Tc(VII) oxidation state, which is relatively unreactive. The preparation of technetium complexes of lower oxidation state Tc(I) to Tc(V) therefore usually requires the addition of a suitable reducing agent such as stannous ion to facilitate complexation. Further suitable reductants are described below.

The metal complex should also preferably exhibit low non-specific blood background.

Thus the present invention relates mainly to diagnostic agents for imaging sites in the mammalian body where the enzyme Factor XIII is activated and blood proteins such as fibrin or collagen are deposited.

The present agents are particularly useful for the diagnostic imaging of the human body. The agents comprise substrates for the enzyme Factor XIIIa which are labelled with a metal complex suitable for external imaging such as a radiometal (for scintigraphy) or a paramagnetic metal ion (for MRI).

5 The metal complex of the present invention has a pendant amino or carboxamide functional group which is available for covalent linking to protein glutamyl carboxamide or lysyl amine groups respectively by the enzyme Factor XIIIa. The intimate relationship of fibrin and Factor XIIIa highlights the potential use of the agents of the present invention for the diagnosis of disease states where there is both fibrin deposition or

10 accumulation and activation of Factor XIII. Increased fibrin deposition is known to be characteristic of diseases such as thrombosis, atherosclerosis, fibrotic liver, and disseminated intravascular coagulation. Fibrin is also deposited at sites of tissue inflammation associated with

15 many disease processes, such as infection, autoimmune disease or cancer. Factor XIII and tissue transglutaminase are activated during known physiological conditions. During apoptosis and generation of new matrix protein structures elevated levels of the enzymes are seen. The present agents may thus also be used for the detection of apoptosis and

20 diseases states such as arthritis where increased matrix protein deposition occurs. Since Factor XIII is activated at the site of interest *in vivo* (i.e. thrombus, embolism etc.) this provides a localisation mechanism for the metal complexes of the present invention. The covalently linked metal complexes can then be imaged externally by radionuclide scintigraphy or

25 magnetic resonance imaging (MRI) hence providing a non-invasive means of diagnosing the disease site.

So far as the therapeutic aspects of this invention are concerned, the inventors have preliminary *in vivo* data (not reported in detail herein) indicating that clots produced in the presence of labelled peptides of the present invention (as per Example 17 below) are smaller

than those produced in the absence of the labelled peptide. On this basis it is proposed that the peptides herein defined, typically containing 4 – 30 e.g. about 10 amino acid residues, are effective as drugs for increasing the rate of clot lysis e.g. by acting as potent inhibitors of fibrin cross linking in 5 clots. Thus the compounds disclosed have possible pharmaceutical uses as thrombolytic or anticoagulant drugs for therapy.

The present invention also relates to kits for the preparation of metal complexes linked to Factor XIIIa substrates. The kits are designed to give sterile products suitable for human administration, e.g. via 10 injection into the bloodstream. Possible embodiments are discussed below. When the detectable moiety is ^{99m}Tc , the kit would comprise a vial containing the free ligand or chelating agent for the metal together with a pharmaceutically acceptable reducing agent such as sodium dithionite, sodium bisulphite, ascorbic acid, formamidine sulphonic acid, stannous ion, 15 Fe(II) or Cu(I), preferably a stannous salt such as stannous chloride or stannous tartrate. Alternatively, the kit could contain a metal complex which, upon addition of the radiometal or paramagnetic metal, undergoes transmetallation (i.e. ligand exchange) giving the desired product. For ^{99m}Tc , the kit is preferably lyophilised and is designed to be reconstituted 20 with sterile ^{99m}Tc -pertechnetate (TcO_4^-) from a ^{99m}Tc radioisotope generator to give a solution suitable for human administration without further manipulation.

The agents of the present invention may also be provided in a unit dose form ready for human injection and could for example be supplied in a pre-filled sterile syringe. When the detectable moiety is a 25 radioactive isotope such as ^{99m}Tc , the syringe containing the unit dose would also be supplied within a syringe shield (to protect the operator from potential radioactive dose).

The above kits or pre-filled syringes may optionally contain 30 further ingredients such as buffers; pharmaceutically acceptable

solubilisers (e.g. cyclodextrins or surfactants such as Pluronic, Tween or phospholipids); pharmaceutically acceptable stabilisers or antioxidants (such as ascorbic acid, gentisic acid or *para*-aminobenzoic acid) or bulking agents for lyophilisation (such as sodium chloride or mannitol).

5 The following examples illustrate the preparation of compounds of the present invention and their use in imaging. The syntheses of particular compounds of the present invention are given in Examples 1-9, their radiolabelling with ^{123}I or $^{99\text{m}}\text{Tc}$ in Examples 10-12. Compound 1 (prior art) is included as a comparative example. Evidence for 10 increased plasma stability *in vitro* is given in Example 13. Evidence for uptake in blood clots *in vitro* and *in vivo* is given in Examples 15 and 17 respectively, with normal rat biodistribution of the radiolabelled compounds reported in Example 16.

15 The *in vitro* plasma stability of ^{123}I -Compound 1 is poor (see Example 13), presumably due to protease activity. The introduction of protecting groups at both the carboxy and amino termini as for radiolabelled Compounds 2-5 and 7-49 confers a substantial increase in plasma stability.

20 The majority of compounds tested exhibit high *in vitro* clot uptake and hence avidity for the clot. The other compounds are of lower potency with compounds 14, 16, 18, 31, 34, 36, 46 and 48 showing significant reduction in uptake. Removal of the Gln residue from position 2 of the α_2 -antiplasmin derived sequences, as in compound 14, causes a large drop in uptake of this tracer, thus strongly suggesting that Gln-2 is an 25 essential amino acid in this sequence type.

Details of the biodistribution in normal rats and in the fresh and aged clot models are given in Examples 16 and 17. The blood clearance rate of these compounds is relatively fast with biological half lives between 1-2 hours. The biodistribution of $^{99\text{m}}\text{Tc}$ -Compound 3 is given 30 as a representative example, in this case the $t_{1/2}$ of 2h is estimated. The

rapid clearance from background tissues such as blood, lung, heart and muscle shows that the agent of the present invention possess favourable pharmacokinetics for imaging and shows their potential as radiodiagnostics. Although some hepatobiliary excretion is seen for these 5 compounds, the main route of excretion is *via* the urinary tract.

Uptake into fresh and aged clots in the rat models for many of the radiolabelled compounds is very good (relative concentration or RC= 5-15), with clot to background tissue ratios very favourable for imaging (>5). Example 18 shows that ^{99m}Tc-Compound 5 is suitable for imaging clots in 10 the rat model.

^{99m}Tc-Compounds 2 - 49 have improved plasma stability compared with ¹²³I-Compound 1 (RC = 1.5), which may be responsible for the improved *in vivo* clot uptake seen with these compounds.

Comparison of the clot uptake results of Example 17 for fresh 15 and aged clots, shows that the present agents exhibit uptake which is constant and independent of the age of the clot. Thus such agents will have improved imaging capability for pre-existing clots, such as those found with pulmonary embolism.

EXPERIMENTAL

In the following table:

Z is benzyloxycarbonyl,

Fmoc is fluorenylmethoxycarbonyl,

Ac is acetyl,

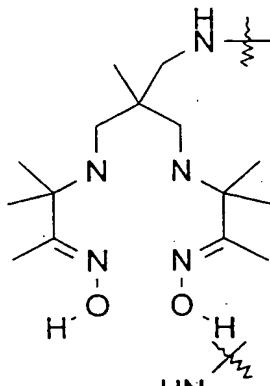
5

10

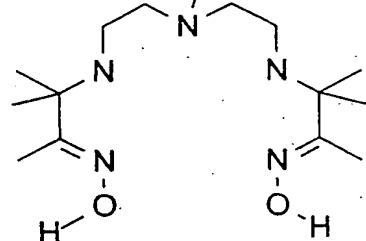
15

20

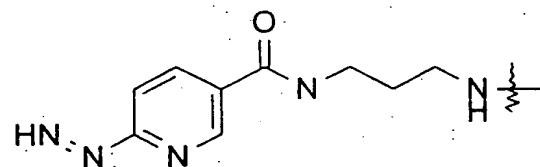
Pn44 is



Pn216 is



Hynic is



Peptide	Compound	MS Theor.	MS Expt.
NQEQQVSPYPTLLK	1	1419.6	1419.7 ^a
Ac-NQEQQVSPYPTLLKG-NH ₂	2	1517.7	1517.7
Ac-NQEQQVSPYPTLLKG-Pn44	3	1816.1	1816.2
Ac-NQEQQVSPYPTLLKG-Hynic	4	1709.7	1710.0
Ac-NQEQQVSPYPTLLKG-Pn216	5	1845.2	1845.2
NQEQQVSPYPTLLKG-Pn216	6	1803.1	1803.3
Z-NQEQQVSPYPTLLKG-Pn44	7	1908.2	1908.1
Z-NQEQQVSPYPTLLKG-Pn216	8	1937.2	1937.2
Fmoc-NQEQQVSPYPTLLKG-Pn216	9	2025.4	2025.4
(CF ₃) ₂ (C ₆ H ₅)-NQEQQVSPYPTLLKG-Pn216	10	2044.2	2045.2
Adamantoyl-NQEQQVSPYPTLLKG-Pn216	11	1966.0	1966.5
Ac-NQEA VSPYPTLLKG-Pn44	12	1759.1	1759.0
Ac-NQEA VSPYPTLLKG-Pn216	13	1788.1	1788.0
Ac-NAEAVSPYPTLLKG-Pn216	14	1766.1	1765.8
Ac-NQQQVSPYPTLLKG-Pn216	15	1844.2	1844.1
Ac-NQG-Pn44	16	656.8	656.6
Ac-NQEQQVSPYPTLLKG-Pn44	17	1908.2	1908.1
Z-NQEQQVSPYPTLLKG-Pn216	18	1481.7	1481.7
Ac-NQEQQVSPYPTLLKG-Pn216	19	1795.2	1795.1
Ac-NQEQQVSP-Nal(2)-TLLKG-Pn216	20	1879.3	1879.3
Ac-NQEQQVSP(pBr-F)TLLKG-Pn216	21	1908.1	1908.9
Ac-NQEQQVSP(I-Y)TLLKG-Pn216	22	1971.2	1972.4
Ac-NQEQQVSP(I ₂ -Y)TLLKG-Pn216	23	2098.2	2099.5
Z-NQEQQVSP(I-Y)TLLKG-Pn216	24	2063.2	2064.4
Z-NQEQQVSP(I ₂ -Y)TLLKG-Pn216	25	2189.2	2190.5
Fmoc-NQEQQVSP(I-Y)TLLKG-Pn216	26	2152.4	2153.4
Fmoc-NQEQQVSP(I ₂ -Y)TLLKG-Pn216	27	2278.4	2279.3
Ac-NQEQQVSPYPTLL(D-K)G-Pn216	28	1845.2	1845.2
Ac-NQEQQVSP(D-Y)TLL(D-K)G-Pn216	29	1845.2	1845.1
Ac-NQEQQV(D-S)P(D-Y)TLL(D-K)G-Pn216	30	1845.2	1845.2

Peptide	Compound	MS Theor.	MS Expt.
Ac-NSEQ(D-V)(D-S)P(D-Y)TLL(D-K)G-Pn216	31	1845.2	1845.1
Ac-(D-N)SEQVSP(D-Y)TLL(D-K)G-Pn216	32	1845.2	1845.0
Ac-NSEQVSP(D-Y)TLL(D-K)βAla-Pn216	33	1859.2	1859.0
Ac-N-CH ₂ NH ₂ -SEQVSP(D-Y)TLL(D-K)G-Pn216	34	1831.2	1831.1
Ac-NSEQ(D-V)(D-S)(D-P)(D-Y)(D-T)(D-L)(D-L)(D-K)G-Pn44	35	1816.1	1815.8
Pn216-CO(CH ₂) ₂ CO-G(D-K)(D-L)(D-L)(D-T)(D-Y)(D-P)(D-S)(D-V)NH ₂	36	1916.2	1916.0
Fmoc-NQQQ(D-V)S(OMe)PLG-Pn216	37	1532.8	1532.7
Pn44-CO(CH ₂) ₂ CO-NSEQVSPYTLKG-NH ₂	38	1887.7	1887.3
Pn216-CO(CH ₂) ₂ CO-NSEQVSPYTLKG-NH ₂	39	1916.2	1916.3
Ac-NSEQVSPYTLKG-(PEG) _{3.4k} -Pn44	40	5200-5600	5401 ^b
Ac-NSEQVSPYTLKG-(PEG) _{10k} -Pn216	41	12400-	12630 ^b
Z-NSEQVSPYAAAG-Pn216	42	1766.0	1765.9
Z-NSEQVSPY(GH ₂) ₁₁ (CO)-Pn216	43	1677.7	1679.8
Cyclo-[NSEQVSPYTLKG]	44	1458.6	1458.3
Ac-LGPGQSKVIG-Pn44	45	1294.6	1294.4
p-EAQIVG-Pn44	46	1023.2	1023.0
Ac-LEFDTQSKNIG-Pn216	47	1733.0	1732.9
Ac-GQDPVKG-Pn216	48	1068.3	1068.0
Ac-YEVHHQKLVFFG-Pn216	49	1872.2	1872.3

All compounds except those denoted were analysed using ES+ mass spectrometry. Those compounds denoted by ^a were analysed by FAB and ^b by MALDI-TOF mass spectrometry.

Example 1: Syntheses of Compounds 1 and 2

The protected peptide Ac-Asn(Trt)-Gln(Trt)-Glu(OtBu)-Gln(Trt)-Val-Ser(tBu)-Pro-Tyr(tBu)-Thr(tBu)-Leu-Leu-Lys(Boc)-Gly-OH was 5 assembled on a 2-chlorotriptyl resin by anchoring Fmoc-Lys(Boc) to the resin, and then successive deprotections/coupling cycles with the appropriate protected amino acids (as described in P. Lloyd-Williams, F. Albericio and E. Girald; *Chemical Approaches to the Synthesis of Peptides and Proteins*, CRC Press, 1997). The title compound was obtained by 10 cleavage using 0.1% TFA in dichloromethane, deprotection and purification by RP-HPLC (System A).

Example 2: Syntheses of Compounds 3-9, 12-21, 28-33, 35, 37, 42 and 45-49

15 The appropriate protected peptide was assembled as in Example 1 with the appropriate protected amino acids. The protected fragment was cleaved from the resin and then coupled with 6-aminomethyl-3,3,6,9,9-pentamethyl-4,8-diazaundecane-2,10-dione dioxime (prepared as described in WO 95/19187), 3,3,11,11-tetramethyl-7-aminoethyl-4,7,10-20 triazatridecane-2,12-dionedioxime (prepared as described in WO 98/31399) or 6-Boc-hydrazinopyridine-3-carboxylic acid N-hydroxysuccinimide ester (prepared as described in US patent 5,206,370) in solution using BOP as a coupling agent. The title compounds were obtained by deprotection in TFA/water/triethylsilane (90/5/5) and purified 25 by RP-HPLC (System A).

Example 3: Syntheses of Compounds 22-27

In an Eppendorf vial compound 5, 8 or 9 (1mg), ammonium acetate buffer (400µl, 0.2M, pH4), sodium iodide (0.5eq, 15mg/10ml in 30 0.1M NaOH) and peracetic acid (1.5 eq, 0.1M solution) were added. The

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reaction mixture was thoroughly mixed for 1 minute and the mono- and di-iodo products were separated and collected by preparative HPLC. The procedure was repeated to give sufficient quantities of the isolated products.

5

Example 4: Syntheses of Compounds 10 and 11

Fmoc-Asn(Trt)-Gln(Trt)-Glu(tBu)-Gln(Trt)-Val-Ser(tBu)-Pro-Tyr(tBu)-Thr(tBu)-Leu-Leu-Lys(Boc)-Gly (200 mg, 0.73 mmol), Pn216 (30 mg, 0.87 mmol) and HBTU (33 mg, 0.87 mmol) were dissolved in anhydrous DMF (2.5 ml). To the solution was added diisopropylethylamine (20 ml, 1.15 mmol) and the reaction mixture was stirred at room for 1.75 hours. The reaction mixture was then treated with piperidine (0.5 ml) and the mixture stirred at room temperature for 2 hours. The product was purified by semi-preparative HPLC to give a white solid (171 mg, 82%);
10 ES⁺-MS: m/z 952.40 (M+3H⁺).

15 1-Adamantanecarboxylic acid or 3,5-bis(trifluoromethyl)benzoic acid (1.5 molar equivalent), the protected peptide Asn(Trt)-Gln(Trt)-Glu(tBu)-Gln(Trt)-Val-Ser(tBu)-Pro-Tyr(tBu)-Thr(tBu)-Leu-Leu-Lys(Boc)-Gly-Pn216 (1 molar equivalent) and HBTU (1.2-1.5 molar equivalent) were dissolved in anhydrous DMF (1 ml). Diisopropylethylamine (11 molar equivalents) was added and the reaction mixture was stirred at room temperature until the reaction was judged complete by HPLC. The protected peptide fragment was then treated with 95% trifluoroacetic acid in CH₂Cl₂. The reaction mixture was stirred at room temperature for 2 to 4 hrs. The product was purified from the reaction mixture by reversed phase HPLC.
20
25

Example 5: Synthesis of Compound 34

30 The protected peptide was synthesised as for example with the exception of the Fmoc-Asn(Trt)-Ψ(CH₂NH)-Gln(Trt)-OH which was

obtained according to the classical methodology for the synthesis of reduced peptide bonds, by reductive amination of Gln(Trt) with Fmoc-Asn(Trt) derived aldehyde (see G. Guichard *et al.*, *Peptide Res.*, **6**(3), 121, (1993) and references therein.

5 The resulting compound was prepared and purified as in example 2.

Example 6: Syntheses of Compounds 36, 38 and 39

10 The protected peptide was synthesised as for example 1 but on a Rink resin. After removal of the glycine N-protection, the peptide was reacted with glutaric anhydride whilst still on the resin. Activation with BOP/HOBt of the glutarate carboxylic acid and coupling with 6-aminomethyl-3,3,6,9,9-pentamethyl-4,8-diazaundecane-2,10-dione dioxime or 3,3,11,11-tetramethyl-7-aminoethyl-4,7,10-triazatridecane-2,12-dionedioxime on the resin gave the protected product. Cleavage with 15 TFA/water (95/5) afforded the crude material which was purified using RP-HPLC (System A).

Example 7: Syntheses of Compounds 40 and 41

20 A solution of the required molecular weight α -N-(*tert*-butoxycarbonyl)-poly(ethylene glycol) amino- ω -succinimidyl carbonate and one molar equivalent of 6-aminomethyl-3,3,6,9,9-pentamethyl-4,8-diazaundecane-2,10-dione dioxime or 3,3,11,11-tetramethyl-7-aminoethyl-4,7,10-triazatridecane-2,12-dionedioxime in anhydrous tetrahydrofuran (5 ml) was refluxed for 5 hours under nitrogen. The reaction mixture was reduced *in vacuo* leading to a white solid which was purified by flash chromatography using isopropanol/ ammonia/ water 10:1:1 to give the title compound as a white solid. The Boc protecting group was removed using 37% HCl added dropwise to an ice-cold solution in methanol. The solution 25 was then stirred at room temperature for 4 hours. The reaction mixture 30 was then stirred at room temperature for 4 hours. The reaction mixture

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was basified to pH ~10 by addition of 4M NaOH (4.18 ml). The product was isolated by semi-preparative HPLC (system D).

5 The solid from above was dissolved in DMF and to it was added the protected peptide fragment. Diisopropylethylamine and HBTU were added and the reaction mixture was stirred at room temperature till completion of the reaction. The product was purified by HPLC (system E) to give a colourless gum.

10 This gum was dissolved in dichloromethane (2 ml) and the solution treated with TFA (0.2 ml) for 5 hours. The reaction was basified with 1M NaOH (2 ml) and volatiles removed *in vacuo*. MeOH (2.5 ml) was added to the residue and the mixture filtered using an Acrodisc filter (LC13 PVDF 0.45 m). The product was isolated from the methanolic solution by HPLC (system E).

15 **Example 8: Synthesis of Compound 43**

12-N-Fmoc-aminododecanoic acid was coupled to ,3,11,11-tetramethyl-7-aminoethyl-4,7,10-triazatridecane-2,12-dionedioxime as described earlier. The Fmoc protecting group was removed by 20% piperidine in DMF and the product purified by HPLC (system F).

20 The above product was coupled with the protected peptide fragment and subsequently deprotected as described above. The product was purified by HPLC (system G).

Example 9: Synthesis of Compound 44

25 The partially deprotected peptide H-Asn(Trt)-Gln(Trt)-Glu(OtBu)-Gln(Trt)-Val-Ser(tBu)-Pro-Tyr(tBu)-Leu-Leu-Lys(Boc)-Gly-OH was assembled on a 2-chlorotrtyl chloride resin, by a Fmoc based strategy, by stepwise elongation with BOP/HOBt. The N-terminal protection was removed by piperidine treatment and partially protected peptide cleaved 30 from the solid support by a 0.5% TFA in dichloromethane solution.

5 Cyclisation was carried out in solution at a 10mM concentration in DMF, with BOP as a condensation reagent, in the presence of solid sodium bicarbonate according to a known methodology (see for instance M. Rodriguez *et al.*, *Int. J. Pept. Protein Res.*, **35**, 441, 1990).

Final deprotection in a mixture of TFA/water/ethane dithiol (90/5/5) afforded the crude title compound which was purified by RP-HPLC (System A).

10 **Example 10: I-123 labelling of Compounds 1-2 and Compound 44**

15 Ammonium acetate buffer (200 μ l, 0.2M, pH 4.0) was added to the ligand solution (20 μ l, 20 μ g), and Na¹²⁷I (10 μ l, 1.5 μ g) in an Eppendorf tube. The solution was mixed thoroughly and Na¹²³I (5-50 μ l, 111MBq) was then added. The solution was mixed thoroughly prior to addition of PAA solution (10 μ l, 0.01M), further mixing followed. The activity of the preparation was measured. In all cases the required product was separated from reaction by-products and unlabelled substrates by HPLC.

20 **Example 11: Tc-99m labelling of Compounds 3, 5-43, 45-49**

25 A 0.1ml aliquot of the compound dissolved in H₂O (1mg/ml) was transferred to a nitrogen-filled 10ml glass vial together with deoxygenated saline (0.9% w/v, 1ml) and 0.035ml aqueous NaOH (0.1M). To this solution was added technetium generator eluate (1ml, approx. 0.4GBq) and then aqueous stannous chloride solution (0.1ml, ca.10 μ g). The labelling pH was 9.0-10.0. Vials were incubated at ambient laboratory temperature (15-25°C) for 30 minutes to effect labelling. The resulting preparation was either diluted to the desired radioactive concentration or HPLC purification was performed (System B) to remove unlabelled starting material and radioactive impurities prior to testing. After purification the

organic solvent was removed under vacuum and the sample was redissolved in about 5ml 0.1M phosphate buffer pH 7.4 to give a working concentration of 6-9MBq/ml. Radiochemical purity was assessed before use by the thin layer chromatography (TLC) system described below:

- 5 i) ITLC SG 2cm x 20cm eluted with 0.9% w/v saline
- ii) Whatman No.1 2cm x 20cm eluted with 50 : 50 v/v acetonitrile : H₂O

The labelled substrates remain at, or close to, the origin in TLC system (i) and move close to the solvent front in system (ii). When analysed by appropriate detection equipment the radiochemical purity is 10 typically in excess of 85% labelled compound.

Example 12: Tc-99m labelling of Compound 4

A 0.1ml aliquot of the compound dissolved in water (1mg/ml) was transferred to nitrogen-filled 10ml glass vial together with tricine 15 dissolved in water (0.5ml, 37.5mg) and phosphinedynetris(benzene sulphonic acid)tris sodium salt dissolved in water (0.1ml, 10mg). To this solution was added technetium generator eluate (1ml, approx 0.4GBq) and then a solution of stannous chloride in 0.1M HCl (0.02ml, ca 2 μ g). The labelling pH was 4.5-5.5. Vials were incubated at 60°C for 30 minutes to 20 effect labelling. Purification and assessment of radiochemical purity was carried out as in Example 10.

Example 13: In vitro plasma stability

To a portion of compound (50 μ l, 10MBq/ml) was added an 25 equal volume of plasma (rat or human) or saline. The mixtures were incubated at 37°C and the stability measured by HPLC (system C) at 0, 30 and 120 minutes. The saline dilution acted as a control.

Compound	Species	% intact at 120 mins
¹²³ I-Cmpd 1 (prior art)	Human	0
¹²³ I-Cmpd 2	Human	98
	Rat	99
^{99m} Tc-Cmpds 3, 4, 5, 7, 12, 16,17; ¹²³ I- Cmpd 44	Human	>90
	Rat	>90
^{99m} Tc-Cmpds 8-11, 13-15,19-21,28-36, 38, 39, 45-49	Rat	>90
^{99m} Tc-Cmpds 22-27	Rat	>60

Example 14: HPLC Systems

Flow Rate: 1ml/min in all systems.

5

System A

Column Waters C18 250x4.5mm. Particle size 4 microns
 Gradient: Elution Profile 10-60% B in 25 min.
 Eluent A: 0.1% aqueous TFA
 10 Eluent B: 0.1% TFA in acetonitrile

System B

Column Waters C18 150x3.9mm. Particle size 4 microns
 Gradient: Elution Profile 0-100% B in 22 min.
 15 Eluent A: 0.1% aqueous TFA
 Eluent B: 0.1% TFA in acetonitrile

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System C

Column Waters C18 150x3.9mm. Particle size 4 microns
Gradient: Elution Profile 0-100% B in 20 min.
Eluent A: 50mM NH₄OAc buffer (pH 5.6)
5 Eluent B: acetonitrile

System D

Column Hamilton PRP-1, 305 mm x 7.0 mm;
Gradient: Elution Profile 0-65% B in 10 min.
10 Eluent A: 5% aqueous ammonia
Eluent B: acetonitrile

System E

Column Hamilton PRP-1, 150 mm x 4.1 mm;
15 Gradient: Elution Profile 0-100% B in 15 min.
Eluent A: 5% aqueous ammonia
Eluent B: acetonitrile

System F

20 Column Polymer Laboratories PLRP-S, 150 mm x 2.5 mm;
Gradient: Elution Profile 0-100% B in 15 min.
Eluent A: 5% aqueous ammonia
Eluent B: acetonitrile

25 System G

Column Hamilton PRP-1, 150 mm x 4.1 mm;
Gradient: Elution Profile 0-100% B in 15 min.
Eluent A: 0.1% aqueous TFA
Eluent B: 0.1% TFA in acetonitrile

Example 15: Incorporation into human plasma clots

Incorporation of radiolabelled substrates into fibrin was investigated by induction of an *in vitro* human plasma clot in the following manner. To a siliconised 5 ml glass vial was added, (a) 800 μ l of 5 *Tris(hydroxymethyl)aminomethane buffered saline pH 7.5* containing calcium chloride (50mM Tris, 150mM sodium chloride, 4mM calcium chloride.), (b) about 40 μ l of physiological salt solution containing 100 units of thrombin per ml, (c) about 400 μ l of human plasma containing the radiolabelled substrate at a concentration of typically 10kBq/ml. To aid 10 induction of clot a roughened glass rod was added to the reaction vial. Control vials were prepared similarly but with the omission of thrombin and calcium chloride.

15 After incubation of the test solution at ambient laboratory temperature (ca. 20°C) for 60 minutes the reaction was discontinued with the addition of about 400 μ l of a cold solution of 33.5mM ethylenediaminetetra-acetic acid disodium salt. Clots were separated from serum by vacuum filtration onto 0.45 μ M nitrocellulose filters (pre-soaked in 1.5% BSA/*tris(hydroxymethyl)aminoethane buffered saline pH 7.5* containing 0.1% Tween 20) and washed with about 2 x 10ml of 20 *tris(hydroxymethyl)aminomethane buffered saline pH 7.5* containing Tween 20 to a final concentration of 0.1%v/v. The proportion of total radioactivity was calculated by counting in suitable detection apparatus.

25 The fraction of radioactivity retained on the filter, after subtraction of the non-specific binding determined from the control, is a measure of incorporation into the filtered clots.

Compound	% retained (with thrombin)	% retained (no thrombin)	% specific uptake
¹²³ I-Cmpd 1	14.6	2.0	12.6
¹²³ I-Cmpd 2	38.6	0.2	38.4
^{99m} Tc-Cmpd 3	39.3	0.3	39.0
^{99m} Tc-Cmpd 4	22.0	0.1	21.0
^{99m} Tc-Cmpd 5	35.5	0.2	35.3
^{99m} Tc-Cmpd 6	68.6	4.3	64.3
^{99m} Tc-Cmpd 7	41.3	0.7	40.6
^{99m} Tc-Cmpd 8	25.9	1.4	24.5
^{99m} Tc-Cmpd 9	44.5	2.7	42.8
^{99m} Tc-Cmpd 10	65.9	0.2	65.7
^{99m} Tc-Cmpd 11	68.1	1.2	66.9
^{99m} Tc-Cmpd 12	34.8	0.3	34.5
^{99m} Tc-Cmpd 13	57.0	0.2	56.8
^{99m} Tc-Cmpd 14	3.3	0.5	2.8
^{99m} Tc-Cmpd 15	55.6	0.4	55.2
^{99m} Tc-Cmpd 16	12.5	0.1	12.4
^{99m} Tc-Cmpd 17	41.3	0.7	40.6
^{99m} Tc-Cmpd 18	9.8	0.1	9.7
^{99m} Tc-Cmpd 19	48.2	0.2	48.0
^{99m} Tc-Cmpd 20	65.0	2.6	62.4
^{99m} Tc-Cmpd 21	60.6	0.5	60.1
^{99m} Tc-Cmpd 22	59.0	0.3	58.7
^{99m} Tc-Cmpd 23	63.8	0.1	63.7
^{99m} Tc-Cmpd 24	56.8	0.4	56.4
^{99m} Tc-Cmpd 25	68.0	4.3	63.7
^{99m} Tc-Cmpd 26	63.3	8.2	55.1
^{99m} Tc-Cmpd 27	Nd	Nd	Nd
^{99m} Tc-Cmpd 28	42.1	0.2	41.9
^{99m} Tc-Cmpd 29	21.1	0.1	21.0
^{99m} Tc-Cmpd 30	14.9	0.2	14.7
^{99m} Tc-Cmpd 31	7.7	0.1	7.6

Compound	% retained (with thrombin)	% retained (no thrombin)	% specific uptake
^{99m} Tc-Cmpd 32	20.2	0.4	19.8
^{99m} Tc-Cmpd 33	63.9	0.1	63.8
^{99m} Tc-Cmpd 34	5.5	0.1	5.4
^{99m} Tc-Cmpd 35	15.9	0.6	15.3
^{99m} Tc-Cmpd 36	8.7	0.1	8.6
^{99m} Tc-Cmpd 37	13.0	0.2	12.8
^{99m} Tc-Cmpd 38	21.0	0.7	20.3
^{99m} Tc-Cmpd 39	23.1	0.1	23.0
^{99m} Tc-Cmpd 40	13.3	0.1	13.2
^{99m} Tc-Cmpd 41	44.5	7.1	37.4
^{99m} Tc-Cmpd 42	14.2	0.2	14.0
^{99m} Tc-Cmpd 43			
¹²³ I-Cmpd 44	14.1	0.3	13.8
^{99m} Tc-Cmpd 45	28.7	0.1	28.6
^{99m} Tc-Cmpd 46	6.3	0.2	6.1
^{99m} Tc-Cmpd 47	49.9	0.1	49.8
^{99m} Tc-Cmpd 48	10.6	0.1	10.5
^{99m} Tc-Cmpd 49	33.1	1.6	31.5

* % retained in plasma clot assay (with thrombin) - % retained in plasma clot assay (no thrombin)

5 Example 16: Normal rat biodistribution

The resolution of a clot image is dependant on the combination of rate of incorporation of the radiopharmaceutical and its blood/tissues clearance rate. For this reason the biodistribution of several compounds has been determined in rats. Male Wistar (100-150g) rats were injected i.v. with 0.1-0.2 ml of radiolabelled tracer solution (8MBq/ml) and dissected at different times post-injection. The %ID in each of the selected tissues was measured. Some animals were kept in metabolism cages to be able to determine the %ID excreted in urine and faeces. The

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dissection times used for the agent were 15, 30, 60, 240 min. Data are shown as mean of %ID (n=3).

^{99m}Tc-Compound 3

5

	15min	30 min	60 min	240 min
Muscle	14.7	10.4	5.1	2.9
Blood	5.3	1.8	1.6	0.6
Kidney	7.6	4.9	4.6	3.4
Urine	14.9	34.3	37.0	42.0
Lung	0.7	0.4	0.3	0.3
Liver	6.2	4.1	4.0	3.0
GI Tract	13.5	15.1	18.0	20.7
Heart	0.2	0.1	0.1	0.04

Example 17: Incorporation into clots induced in a rat model

Rat Inferior vena cava model (IVC)

The rats (Male Wistar, 250-350g) were anaesthetised with

10 15% urethane. After laparotomy, the vena cava was isolated and freed of surrounding fat tissue. A platinum wire (1.5cm x 0.5mm) was inserted into the inferior vena cava and 5 min post surgery 0.4ml of ellagic acid (1.2×10^4 M) was injected intravenously through the femoral vein previously canulated, and the clot was allowed to form. The average

15 weight of the clots formed in this model was around 27mg, n=32, (5-50mg range). The compounds were injected 5 min (fresh clot) and 60 min (aged clot) post-induction. After 60 min the animals were sacrificed and the clot removed, weighed and counted. Other tissues e.g. blood, lung, heart, were also dissected and counted. The uptake of tracer into the clot was

20 determined as the relative concentration (cpm/g of clot by dose/g animal)

and clot to background tissue.

Results:

5 **Fresh Clots**

Compound	%id/g	Rel. Conc.	Clot/blood	Clot/lung	Clot/ heart	Clot/ liver
¹²³ I-Cmpd 1	0.5±0.1	1.6±0.4	1	nd	Nd	nd
¹²³ I-Cmpd 2	4.9±0.8	14.5±2.2	7	10	19	21
^{99m} Tc-Cmpd 3	1.4±0.4	5.1±1.2	10	8	17	6
^{99m} Tc-Cmpd 4	2.0±0.6	6.5±2.0	6	8	15	20
^{99m} Tc-Cmpd 5	6.0±2.1	16±5.8	21	23	51	43
^{99m} Tc-Cmpd 7	4.8±1.3	14.5±4.4	15	11	21	6
^{99m} Tc-Cmpd 8	6.7±1.7	21.0±6.8	14	18	41	21
^{99m} Tc-Cmpd 9	8.8±3.6	24.8±9.6	13	12	32	13
^{99m} Tc-Cmpd 10	2.9±0.5	6.5±1.2	4	5	10	5
^{99m} Tc-Cmpd 11	1.1±0.4	3.3±1.1	10	11	24	11
^{99m} Tc-Cmpd 12	7.3±4.1	19.8±11.0	20	17	31	8
^{99m} Tc-Cmpd 13	3.4±0.7	7.9±1.6	7	8	18	18
^{99m} Tc-Cmpd 14	0.5±0.1	1.2±0.2	2	1.5	3	0.7
^{99m} Tc-Cmpd 15	3.6±1.0	8.3±2.2	4	6	12	11
^{99m} Tc-Cmpd 16	0.3±0.1	1.0±0.2	3	3	8	0.8
^{99m} Tc-Cmpd 17	1.2±0.7	3.7±2.3	7	12	18	5
^{99m} Tc-Cmpd 19	2.5±0.3	7.1±1.2	3	7	29	17
^{99m} Tc-Cmpd 20	2.3±0.6	6.5±1.3	12	11	30	18
^{99m} Tc-Cmpd 21	2.3±1.3	7.4±4.0	11	16	28	13
^{99m} Tc-Cmpd 22	3.8±1.6	13.0±5.5	11	13	28	25
^{99m} Tc-Cmpd 23	7.6±1.8	18.2±4.1	6	6	17	21
^{99m} Tc-Cmpd 24	4.0±2.2	11.1±6.7	10	10	21	11
^{99m} Tc-Cmpd 26	14.1±10	41.4±28	16	11	38	16
^{99m} Tc-Cmpd 28	3.5±2.0	9.7±5.0	13	19	31	35
^{99m} Tc-Cmpd 29	4.7±1.9	13.0±4.9	14	20	44	44
^{99m} Tc-Cmpd 31	0.3±0.2	0.9±0.6	0.6	1	2	3

- 31 -

Compound	%id/g	Rel. Conc.	Clot/blood	Clot/lung	Clot/ heart	Clot/ liver
^{99m} Tc-Cmpd 32	1.7±0.7	4.6±1.9	10	11	26	27
^{99m} Tc-Cmpd 33	2.7±0.2	7.8±0.6	11	16	32	28
^{99m} Tc-Cmpd 34	0.5±0.2	1.8±0.5	2	3	6	8
^{99m} Tc-Cmpd 35	1.2±0.2	3.7±0.8	2	2	4	2
^{99m} Tc-Cmpd 36	0.2±0.1	0.7±0.3	1	1	3	4
^{99m} Tc-Cmpd 37	0.4±0.2	0.9±0.4	0.6	0.7	1	1
^{99m} Tc-Cmpd 38	1.9±0.5	5.4±1.5	5	6	15	3
^{99m} Tc-Cmpd 39	2.5±0.3	9.0±1.1	9	14	29	31
^{99m} Tc-Cmpd 41	0.7±0.1	2.2±0.3	3	4	6	8
^{99m} Tc-Cmpd 42	0.3±0.1	0.3±0.2	2	0.3	1	63
¹²³ I-Cmpd 44	0.4±0.1	1.1±0.2	0.9	1.5	1	2
^{99m} Tc-Cmpd 45	2.0±0.5	5.5±1.3	6	5	10	3
^{99m} Tc-Cmpd 46	0.8±0.4	2.0±0.7	5	3	6	1
^{99m} Tc-Cmpd 47	0.8±0.7	2.2±2.0	0.8	1	3	1
^{99m} Tc-Cmpd 48	0.3±0.2	1.0±0.5	0.6	0.9	2	3
^{99m} Tc-Cmpd 49	2.6±1.1	8.5±3.7	4	6	16	4

Rel. Conc.(RC) = $\frac{\text{%id/g of clot}}{\text{%id/g in rest of body}}$

5 Aged Clots

Compound	%id/g	Rel. Conc.	Clot/blood	Clot/lung	Clot/ heart	Clot/ liver
¹²³ I-Cmpd 2	5.5±1.7	14.5±3.9	24	12	23	20
^{99m} Tc-Cmpd 3	2.1±0.8	6.2±2.2	8	11	23	5
^{99m} Tc-Cmpd 4	1.2±1.1	4.1±4.0	8	10	19	19
^{99m} Tc-Cmpd 5	3.6±1.7	11±5.1	13	31	33	24
^{99m} Tc-Cmpd 7	2.1±0.3	6.5±1.3	9	7	15	3
^{99m} Tc-Cmpd 8	5.3±1.3	16.4±4.3	10	9	24	16
^{99m} Tc-Cmpd 9	3.7±0.4	11.4±1.5	8	7	21	6
^{99m} Tc-Cmpd 10	2.9±1.4	6.3±2.7	6	6	16	5
^{99m} Tc-Cmpd 11	0.4±0.3	1.2±0.8	5	6	13	4
^{99m} Tc-Cmpd 12	4.1±1.2	11.2±3.4	19	15	75	7

Compound	%id/g	Rel. Conc.	Clot/blood	Clot/lung	Clot/ heart	Clot/ liver
^{99m} Tc-Cmpd 13	4.5±1.5	10.4±3.0	6	9	29	19
^{99m} Tc-Cmpd 14	0.6±0.4	1.4±0.8	1.4	1.3	2	0.7
^{99m} Tc-Cmpd 15	3.4±0.6	7.6±1.6	3	0.6	1	nd
^{99m} Tc-Cmpd 17	0.8±0.1	2.7±0.6	3	5	7	3
^{99m} Tc-Cmpd 19	3.2±2.3	9.1±7.1	14	5	13	6
^{99m} Tc-Cmpd 20	1.7±0.5	4.9±1.4	10	14	36	23
^{99m} Tc-Cmpd 21	2.7±2.3	8.5±6.8	31	13	32	31
^{99m} Tc-Cmpd 22	6.9±2.5	22.7±7.4	11	1	3	4
^{99m} Tc-Cmpd 23	7.5±2.5	17.6±4.6	13	1	1	1
^{99m} Tc-Cmpd 24	4.1±2.5	11.9±8.0	15	20	40	37
^{99m} Tc-Cmpd 26	4.0±0.9	12.9±3.5	7	12	31	54
^{99m} Tc-Cmpd 28	4.5±1.1	13.1±3.4	17	2	3	1
^{99m} Tc-Cmpd 29	3.8±0.4	10.0±1.7	11	30	67	29
^{99m} Tc-Cmpd 31	0.2±0.1	0.6±0.2	0.6	4	9	2
^{99m} Tc-Cmpd 32	0.7±0.1	2.0±0.4	3	15	43	21
^{99m} Tc-Cmpd 33	2.2±0.6	6.7±1.6	16	9	24	16
^{99m} Tc-Cmpd 34	0.4±0.1	1.4±0.4	3	-	-	6
^{99m} Tc-Cmpd 35	0.6±0.1	1.5±0.4	1	0.5	1	2
^{99m} Tc-Cmpd 36	0.1±0.02	0.2±0.1	0.6	10	18	4
^{99m} Tc-Cmpd 37	0.2±0.03	0.5±0.1	0.4	1	2	9
^{99m} Tc-Cmpd 38	0.8±0.3	2.4±0.7	3	0.5	1	2
^{99m} Tc-Cmpd 39	2.4±0.3	8.2±1.1	15	12	43	11
^{99m} Tc-Cmpd 41	0.4±0.1	1.1±0.3	1	6	13	4
^{99m} Tc-Cmpd 42	0.4±0.1	1.3±0.3	5	7	21	6
¹²³ I-Cmpd 44	0.5±0.03	1.2±0.1	0.9	2	1	1
^{99m} Tc-Cmpd 45	2.0±0.8	5.7±2.3	8	5	11	3
^{99m} Tc-Cmpd 46	0.5±0.2	1.3±0.6	4	3	5	1
^{99m} Tc-Cmpd 47	0.5±0.3	1.5±0.8	1	24	46	44
^{99m} Tc-Cmpd 48	0.2±0.04	0.8±0.2	1	109	32	26
^{99m} Tc-Cmpd 49	1.2±0.5	3.9±1.6	4	9	29	19

Example 18: Imaging of clots induced in a rat model

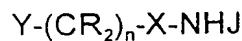
Clots were induced in male Wistar rats (250-350g) as described in Example 17, with the exception that for these experiments the platinum wire was located in the jugular vein. The compounds were 5 injected 60 min post injection and planar images acquired between 15-180 min p.i. A Park medical Isocam I gamma camera was used for these experiments, 300K or 150K counts were of the thorax were collected using a LEUHR or a LEPH collimator. Clots were visualised from 15 min p.i. Reaching the highest clot to background ratio at 180 min p.i. due to rapid 10 clearance of the compound.

Abbreviations

Ac	Acetyl
Boc	tert-butyloxycarbonyl
15 Cmpd	compound
DMF	dimethylformamide
ES	electrospray
FAB	fast atom bombardment
Fmoc	fluorenylmethoxycarbonyl
20 HPLC	high performance liquid chromatography
MALDI-TOF	matrix assisted laser desorption ionisation – time of flight
Nal	napthylalanine
RCP	radiochemical purity
25 RP-HPLC	reverse phase high performance liquid chromatography
TFA	trifluoroacetic acid
TLC	thin layer chromatography

CLAIMS

1. A compound of formula:



5 where:

X is C=O or CR₂;

n is an integer of value 1 to 6;

Y is L(A)_m- or R¹R²CR- where L is a metal complexing agent,

A is -CR₂- , -CR=CR- , -C≡C- , -NRCO- , -CONR- , -SO₂NR- ,

10 -NRSO₂- , -CR₂OCR₂- , -CR₂SCR₂- , -CR₂NRCR₂- , a C₄₋₈

cycloheteroalkylene group, a C₄₋₈ cycloalkylene group, a C₅₋₁₂ arylene

group, a C₃₋₁₂ heteroarylene group or a polyalkyleneglycol, polylactic acid
or polyglycolic acid moiety;

m is an integer of value 0 to 10;

15 where one of R¹ and R² is -NH(B)_pZ¹ and the other is

-CO(B)_qZ² where

p and q are integers of value 0 to 45, and

each B is independently chosen from Q or an amino acid
residue,

20 where Q is a cyclic peptide;

Z¹ and Z² are protecting groups;

J and each R group are independently chosen from H, C₁₋₄
alkyl, C₁₋₄ alkenyl, C₁₋₄ alkynyl, C₁₋₄ alkoxyalkyl or C₁₋₄ hydroxyalkyl;

with the provisos that:

25 (i) the total number of amino acid residues in the R¹ and R²
groups does not exceed 45;

(ii) when X is CR₂, then Y is -CRR¹R² and Z² is a metal
complexing agent;

(iii) when Y is -CRR¹R² then at least one of R¹ and R² bears at
30 least one detectable moiety.

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2. The compound of claim 1 where R¹ or R² includes one or more peptide fragments of α_2 -antiplasmin, fibronectin, beta-casein, tetanus, amyloid, trappin and polyglutamine residues, said peptide fragment containing at least three amino acid residues.

5

3. The compound of claim 2 where the peptide fragment is from α_2 -antiplasmin.

4. The compound of claim 3 where the amino acid in the 2-position from the peptide N-terminus is glutamine.

10 5. The compound of claims 1 to 4 where J is H.

6. The compound of claim 5 of formula:

15 Y-(CR₂)_x-(CH₂)₂CONH₂ or Y-(CR₂)_y-(CH₂)₄NH₂

where x is an integer of value 0 to 4, and y is an integer of value 0 to 3.

20 7. The compound of any one of claims 1 to 6 where Y is -CRR¹R².

8. The compound of any one of claims 1 to 7 where at least one of Z¹ and Z² is a metal complexing agent.

25 9. The compound of claim 8 where Z² is a metal complexing agent and Z¹ is not a metal complexing agent.

10. A metal complex of the compounds of claim 8 or claim 9.

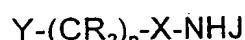
30 11. The metal complex of claim 10 where the metal is a radiometal.

12. The radiometal complex of claim 11 where the radiometal is ^{99m}Tc .

13. A preparation for human administration comprising the 5 compound of any one of claims 1 to 12.

14. A kit comprising the compound of any one of claims 1 to 9 useful in the preparation of the metal complexes of any one of claims 10 to 12.

10 15. Use for the diagnosis of sites of thrombosis or embolism of a compound of formula:



where:

15 X is $\text{C}=\text{O}$ or CR_2 ;

n is an integer of value 1 to 6;

Y is $\text{L}(\text{A})_m-$ or $\text{R}^1\text{R}^2\text{CR}-$ where L is a metal complexing agent,

A is $-\text{CR}_2-$, $-\text{CR}=\text{CR}-$, $-\text{C}\equiv\text{C}-$, $-\text{NRCO}-$, $-\text{CONR}-$, $-\text{SO}_2\text{NR}-$,

$-\text{NRSO}_2-$, $-\text{CR}_2\text{OCR}_2-$, $-\text{CR}_2\text{SCR}_2-$, $-\text{CR}_2\text{NRCR}_2-$, a C_{4-8}

20 cycloheteroalkylene group, a C_{4-8} cycloalkylene group, a C_{5-12} arylene group, a C_{3-12} heteroarylene group or a polyalkyleneglycol, polylactic acid or polyglycolic acid moiety;

m is an integer of value 0 to 10;

where one of R^1 and R^2 is $-\text{NH}(\text{B})_p\text{Z}^1$ and the other is

25 $-\text{CO}(\text{B})_q\text{Z}^2$ where

p and q are integers of value 0 to 45, and

each B is independently chosen from Q or an amino acid residue,

where Q is a cyclic peptide;

30 Z^1 and Z^2 are protecting groups;

J and each R group are independently chosen from H, C₁₋₄ alkyl, C₁₋₄ alkenyl, C₁₋₄ alkynyl, C₁₋₄ alkoxyalkyl or C₁₋₄ hydroxyalkyl; with the provisos that:

- (i) the total number of amino acid residues in the R¹ and R² groups does not exceed 45;
- (ii) when X is CR₂, then Y is -CRR¹R²;
- (iii) when Y is -CRR¹R² then at least one of R¹ and R² bears at least one detectable moiety.

10 16. Use for the diagnosis of sites of thrombosis or embolism of a radiometal complex of the compound defined in claim 13, wherein at least one of Z¹ and Z² is a metal complexing agent.

15 17. A peptide fragment of α_2 -antiplasmin, fibronectin, beta-casein, tetanus, amyloid, trappin or polyglutamine, said peptide fragment containing 3 – 45 amino acid residues and carrying a terminal metal complexing agent.

20 18. The peptide fragment of claim 17, wherein the metal complexing agent is at the carboxy terminus.

19. A metal complex of the peptide fragment of claim 17 or claim 18.

Figure 1: Imaging with 99m Tc-Compound 5.

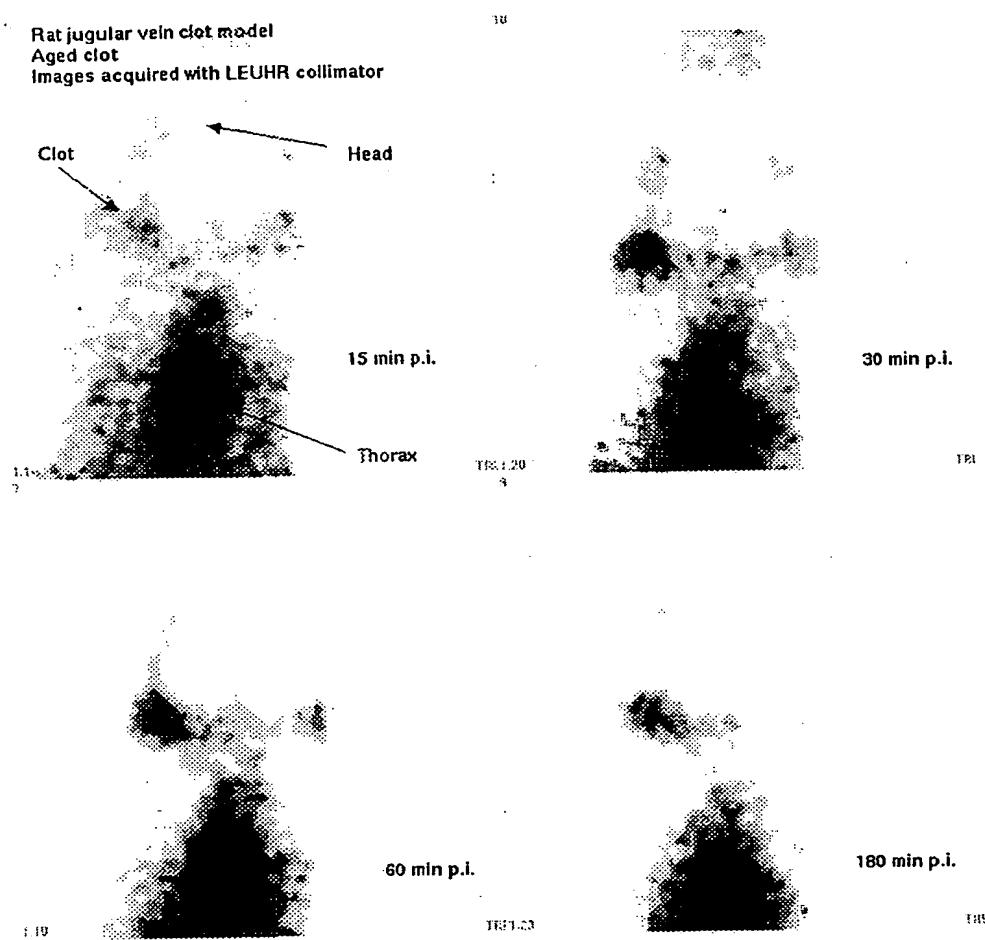
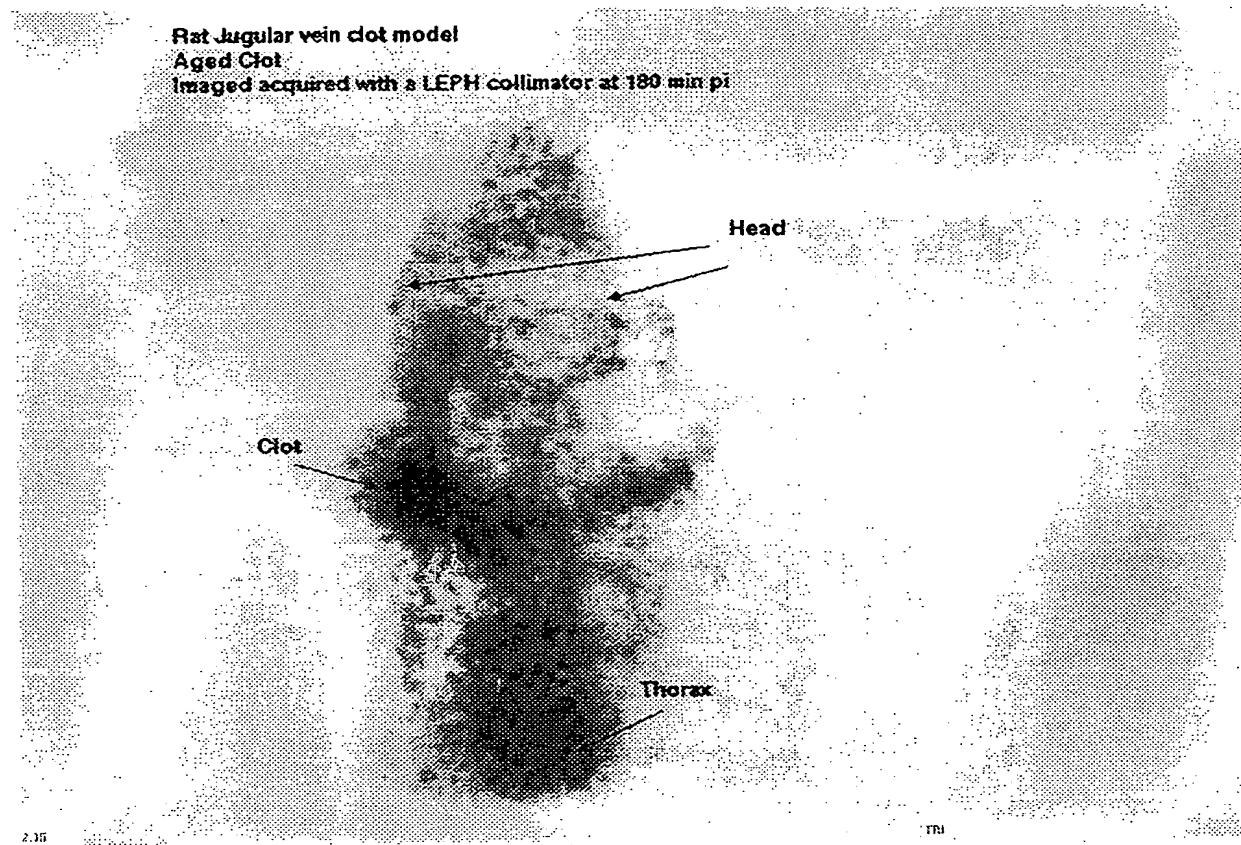


Figure 2: Imaging with 99m Tc-Compound 5.



INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/01550

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C07K14/00 C07K14/81 C07K14/78 A61K51/08 G01N33/60

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 98 31399 A (MENDIZABAL MARIVI ; WILSON IAN ANDREW (GB); GIBSON ALEX (GB); CHAMP) 23 July 1998 (1998-07-23) the whole document ---	1-19
X	WO 89 00051 A (CYTRX BIOPPOOL LTD) 12 January 1989 (1989-01-12) cited in the application page 9; claims ---	1-19
X	WO 91 17765 A (BIO TECHNOLOGY GENERAL CORP) 28 November 1991 (1991-11-28) page 7, paragraph 1 page 31, line 10 - line 15; claims ---	1, 2, 5-19
X	EP 0 121 995 A (KOWA CO) 17 October 1984 (1984-10-17) page 4, line 23 ---	1, 2, 5-19
		-/-



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

23 August 1999

27/08/1999

Name and mailing address of the ISA

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Cervigni, S

INTERNATIONAL SEARCH REPORT

Inte Application No

PCT/GB 99/01550

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 629 617 A (SQUIBB BRISTOL MYERS CO) 21 December 1994 (1994-12-21) page 2-3 page 22 ---	1,5-16
X	WO 93 12819 A (RHOMED INC) 8 July 1993 (1993-07-08) page 1 page 9-11 page 16, line 9 ---	1,5-16
X	D.A. PEARSON ET AL.: "Thrombus imaging using technetium-99m-labeled high-potency GPIIb/IIIa receptor antagonists." J. MED. CHEM., vol. 39, 1996, pages 1372-1382, XP002080979 See scheme 1 figure 1 ---	1,5-16
X	J. LISTER-JAMES ET AL.: "Pre-clinical evaluation of Technetium-99m platelet receptor-binding peptide" J. NUCLEAR MEDICINE, vol. 38, no. 1, January 1997 (1997-01), pages 105-111, XP002080980 figure 2 ---	1,5-16
X	S. VALLABHAJOSULA ET AL.: "Atherosclerosis: imaging techniques and the evolving role of nuclear medicine" J. NUCLEAR MEDICINE, vol. 38, no. 11, November 1997 (1997-11), pages 1788-1796, XP002080981 the whole document page 1794, column 1, last paragraph -----	1,5-16

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 99/01550

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 15, 16

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 15 and 16 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. Claims Nos.:

because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/01550

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		AU 678001	B	15-05-1997
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		CA 2125895	A	16-12-1994
		CN 1099388	A	01-03-1995
		CZ 9401455	A	17-05-1995
		DE 69409870	D	04-06-1998
		DE 69409870	T	10-12-1998
		ES 2115805	T	01-07-1998
		FI 942795	A	16-12-1994
		JP 7089922	A	04-04-1995
		NO 942231	A	16-12-1994
		PL 303843	A	27-12-1994
		US 5608110	A	04-03-1997
		US 5656254	A	12-08-1997
		US 5627286	A	06-05-1997
		US 5741912	A	21-04-1998
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